Extracorporeal perfusion of isolated organs of large animals - Bridging the gap between in vitro and in vivo studies

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Review Article

Extracorporeal Perfusion of Isolated Organs of Large Animals – Bridging the Gap Between *in vitro* and *in vivo* Studies

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Summary
Since the early 20th century extracorporeal perfusion of large animal organs has been used to investigate a broad variety of research questions, thereby overcoming common draw backs of in vitro studies without suffering from ethical concerns associated with live animal research. The technique is in accordance with the 3R principles and represents an excellent opportunity to investigate in detail the physiology of organs under standardised conditions. It is also suitable for the translation of basic pre-clinical research into a more relevant arena prior to or avoiding altogether live animal research. Furthermore, organ perfusion has also been an important tool in developing methods of organ preservation for transplantation surgery. Yet due to the nature of the experiments only short term observations can be made and while cells are still exposed to their regional secretome, the whole organ itself is isolated from the body and correlations between organ-systems cannot be taken into consideration. This review gives an overview over the history of extracorporeal perfusion of large animal organs and limbs highlighting major achievements in the field and discussing different experimental set-ups. Advantages and limitations of this technique are presented. Prospective future research perspectives, which might include tracking of specific cell types and study of their distinct behaviour towards different stimuli, are given to illustrate the relevance of this method.

Keywords: Extracorporeal perfusion, large animal, research model, isolated perfused organ

1 Introduction[1]

Different scientific approaches are adopted to investigate research questions in physiology or pathophysiology; their spectrum reaches from cell cultures on one side to in vivo experiments on the other. There are numerous advantages associated with in vitro cell culture based research. The population of cells is very homogenous if they are sourced from the same individual and tissue. Experiments can be carried out in a strictly controlled and replicable environment, and costs are comparatively low allowing for multiple replicates. This can rarely be achieved in ex or in vivo set-ups due to higher costs, tissue availability and ethical concerns. Cultured cell lines do allow research on species specific cells; this is of particular advantage for modelling human disease, especially if primary cell lines closely resemble the situation in the donor. However primary (human) cell lines may not always be available for the disease of interest. In vivo and ex vivo experiments are usually limited to animal species and extrapolation from such research to the human disease is often difficult. Additional disadvantages of cell culture experiments include the isolation of cells from their natural environment and the resulting lack of physiological cell-to-cell contact and mediators (Caron et al., 2012). On the other hand, in vivo experiments best reflect the complex physiological environment, and accordingly they are nearly universally used in the later stages of clinical research to validate earlier inferences from in vitro studies. However, there are notable individual differences even between genetically similar animals, thus requiring research on a considerable number of animals and consequentlylong and costly experimental set-ups affecting animal well-being to a greater extent (Gruber et al., 2004). One of the methods aiming to

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[1]**List of abbreviations**
3R, Reduce, refine, replace; ATP, adenosine triphosphate; CD11b, cluster of differentiation molecule 11b; CPB, cardiopulmonary bypass; ECP LA, extracorporeal perfusion of large animal organs; HLA-DR, human leukocyte antigen-antigen Drelated (receptor); ILP, isolated limb perfusion; LDH, lactate dehydrogenase; Na+/K+ ATPase, sodium-potassium adenosine triphosphatase; NADH, reduced form of nicotinamide adenine; dinucleotide; pCO_2, partial pressure of carbon dioxide; pO_2, Partial pressure of oxygen; SO_2, oxygen saturation

close the gap between the two aforementioned techniques is the use of ex vivo tissue explants. In explant cultures, small pieces of tissue are harvested and cultured as a whole; this retains both the microenvironment of the resident cell populations as well as 3D structure and natural matrix.However, explant cultures are lacking blood circulation and regional secretome exposure.In addition to thatthe process of harvesting such tissue explants has been shown to induce inflammationdiminishing the ability to accurately predict in vivo outcomes (McCoy, 2015).Compared to tissue explants, extracorporeal organ perfusionfurther approximates the in vivo environment, with organ specific cells maintained in their natural 3D structure and providing a functional blood supply. By using abattoir derived specimens, live animal research may be avoided or at leastfurther reduced given the possibility of detailed management of perfusion parameters allowinggreater refinement of later in vivo experiments.However, the genetic heterogeneity of organ donors may also influence experimental data variability, and as with ex vivo tissue explants, inflammatory changes may occur during the harvest, transport, and storage of organs, as well as during the extracorporeal perfusion as demonstrated during cardiopulmonary bypass surgery where blood is pumped through an extracorporeal system (Butler et al., 1993).Isolated perfused organs retain their functional blood supply but they are missing the complex interplay of organs and metabolic features of a live organism, e.g. hepatic metabolism, nociception or febrile responses. Isolated perfused organs are therefore not reflecting the in vivo situation in its full complexity.

Historically, extracorporeal perfusion of large animal organs(ECP LA) has been successfully performedfor over a century. The possibility to harvest organs at an abattoir without affecting the commercial use of animals, has allowed large studies to be carried out using ECP LA with no effect on animal life or well-being; e.g. in one example 492 organs (hearts n=191, lungs n=108, livers n=48, kidneys n=145) were perfused and analysed (Grosse-Siestrup et al., 2002a).Although it is possible to harvest multiple organs at the same time, requirements for staff and equipment commonly limits experiments to the simultaneous perfusion of one or two organs.

In veterinary science, isolated limb perfusion (ILP) has facilitated new insights into the clinical picture of laminitis(Wüstenberg, 2006; Patan-Zugaj et al., 2012, 2014) a severely debilitating, painful and potentially life threatening equine and bovine disease.Due to its significant impact on animal well-being and the complexityof potential causative agents and risk factors continued research efforts are unquestionably needed.Whilefew studies have used live animals, the justification to induce such a painful condition in live animals may be challenged. The sample sizes required for scientific validity andachieved in studies utilizing organ perfusion methods (e.g. n=10 (Patan-Zugaj et al., 2012), n=14 (Patan-Zugaj et al., 2014)) would certainly not have been justifiable in vivo.

Although the focus of this review is on the perfusion of organs of large animals, it should be mentioned that the same technique has been very successfully applied in small animals. Extracorporeal organ perfusion in small animal species was carried out predominantly in rats (Bounakta et al., 2017) but also in rabbits (VanGiesen et al., 1983), Guinea pigs (Kleber, 1983), dogs (Kitaguchi et al., 1979), cats (Hebb and Linzell, 1951), as well as mice (Wang and Wang, 2005); rat organs perfused comprised hearts (Yue et al., 2000), liver and intestines (Windmueller et al., 1973), kidney (Nishiitsutsuji-Uwo et al., 1967), limbs (Hicks et al., 1980), liver (Hager and Kenney, 1968), and lung (Seibert et al., 1993).

The aim of this review is to describe the historical development of large animal organ perfusion for research, to compare and contrast the methods currently used in ECP LA and to indicate which research interests are best suited for which organ perfusion technique. Furthermore, the most pertinent findings obtained applying this methodologywill be presented.

2 Methodologies used for Extracorporeal Perfusion of Large Animal Organs

English and German articles used for this review were identified using the search engines PubMed and Google Scholar. Keywords used were “perfusion/ perfused”, “isolated” “extracorporeal”, “preservation”, “ex vivo” and “animal model”. Additional materials referenced in the articles initially identified were also included where suitable. The authors focused on research conducted in large animal organs (bovine, porcine, equine, ovine, and caprine). In case the same conclusion was drawn in multiple articles a selection of references was used.

In this section of the review, the following nomenclature will be used: the term “perfusion” is used for a technique in which organs are separated from the body’s blood circulation but maintained under physiological conditions using an extracorporeal artificial circuit. Specimens used in these experiments are denoted as “organs” which includes internal organs as well as limbs, muscles, skin, and udder unless otherwise specified. The fluid serving as the blood equivalent in perfusions will be referred to as “perfusate” and the pressure, flow rate and temperature the perfusate is administered at, are termed “perfusion pressure, - flow and – temperature” respectively.

In the field of perfusion numerous set-ups have been designed and individually tailored to fit specific requirements. Next to the hardware set-up, a multitude of different variables have to be defined as well, such as perfusion flow and pressure, perfusate composition and oxygenation. It is difficult to compare findings obtained in different species and organs on the basis of varying experimental techniques and scientific aims as isolated organ perfusion represents a complex interaction between numerous different factors that cannot be fully assessed in isolation. However, an outline of commonly applied methods in ECP LA experiments may still serve to demonstrate the range of possibilities and inform decisions regarding future use of this methodology.

The authors propose that as a minimum the following information should be reported for extracorporeal perfusion experiments in order to allow other researchers to comprehend the conducted experiments and potentially replicate results: donor selection, organ collection and transport, perfusion fluid (oxygenation system, perfusion flow and pressure, temperature, type, additives), and viability measurements, such as oedema formation, oxygen uptake and glucose consumption. Parameters indicative of cell death such as levels of potassium and lactate dehydrogenase (LDH) in the used perfusate is also recommended. Histology and organ specific parameters are of additional benefit.

2.1 Donor Selection

Up to date, as research has predominantly focused on physiology aspects or organ preservation techniques specimens from healthy large animals have largely been used. Specimens of healthy individuals can be sourced from abattoirs thereby supporting 3R principles (reduce, refine, replace). The collection of naturally diseased organs represents a promising future step, however it requires greater flexibility of the research team, as the availability of such organs is typically difficult to predict, as diseased animals will not commonly be presented to local abattoirs. In the interest of sufficient preparation time and availability of staff this approach may therefore proof challenging. Alternatively, the use of organs from animals that have had disease induced in the course of (other) experiments, and have reached their study end-point may allow greater use of available resources.In most terminal experiments, not all organs are used, and those non-central to the disease investigated are simply discarded allowing these organs to be harvested. This is certainly an avenue worth pursuing as such organs may allow modellingof advanced disease stages, when the original animal experiment as such has been concluded. However, in order to effectively utilize this resource, close links between research groups are required. Finally, induction of disease in live animals for the sole purpose of using their organs post mortem could also be considered. While this would still reduce the burden on animal well-being and facilitate structured and controlled experiments it negates one of the core 3R principles: The replacement of animal use altogether.

The decision whether to use euthanized or slaughtered animals would depend on the perfusion set-up and research question. In euthanized animals, the available blood volume for perfusion is smaller, as animals are not stunned and exsanguinatedrendering autologous blood perfusion difficult to achieve. Moreover, potential drug interactions between euthanasia solutions (typically barbiturates) and organ performance during perfusions remain a concern. To the authors' knowledge these questions have not been addressed so far and would require answering to fully promote use of naturally diseased organs harvested after euthanasia.

2.2 Organ Collection and Transport

Organs should be harvested as soon as possible after death, to avoid excessive ischemia/ reperfusion injury (Blaisdell, 2002). As tissues show variable susceptibility towards ischemia, the critical time window varies depending on the organs harvested(Steinau, 2013). Limbs represent a particular challenge, as due to the inclusion of skeletal muscle, specimens are particularly sensitive to ischemia with a critical time window of four hours only(Blaisdell, 2002). Arteries used for perfusion have to be cannulated swiftly, and larger arteries not used for perfusion have to be occluded to reduce retrograde perfusateflow. If a closed system is intended, major veins also requirecannulation withthe remaining veins equally occluded. In open systems veins remain open and the venous return can drip freely into a reservoir or sampling container.Once catheters have been placed, specimens should be either connected to the perfusion circuit or, if transportation of the specimen is required, flushed with cold preservation solution. Either way the first fluid used for perfusion should always contain an anticoagulant to prevent thrombus formation. If temporary storage or transport of the organ is necessary, the time of warm ischemia should be kept as short as possible. Therefore after the cold flush, specimens should also be cooled externally to further reduce the metabolism (Patan et al., 2009). This is best achieved by wrapping specimens in electrolyte solution soaked swabs or by complete immersion in a plastic bag and subsequent storage on ice(VanGiesen et al., 1983). While this is suitable for many internal organs, practical considerations might not always allow this method, in particular when skin contamination of limb specimens may be a concern. The ischemic phase of the organ is a concern in perfusion experiments. The underlying mechanism of anoxic cell injury of O_2-dependent cells during ischemia is a drop in adenosine triphosphate (ATP) synthesis and oxidative phosphorylation. This leads to detrimental intracellular changes (disruption of homeostasis, increased membrane permeability, activation of hydrolases) and ultimately to cell death. While this process is basically the same in warm and cold ischemia, it is markedly slower if organs are kept between four and zero degree Celsius (De Groot and Rauen, 2007). Upon reperfusion of the organ an inflammatory response is triggered by cell debris and/or altered tissue matrix as a result of anoxic cell injury; this may also be triggered by cold induced iron ions dependent apoptosis (e.g. endothelial cells, renal tubular cells, hepatocytes). So despite showing the same ischemic injury (at different rates), reperfusion injury and inflammatory response are distinct after warm or cold ischemia respectively (De Groot and Rauen, 2007). Complex preservation solutions are aiming to minimise the problems associated with reperfusion injury by protective compounds such as iron chelators (to reduce cold induced apoptosis) by low levels of sodium (to re-establish cell homeostasis) (De Groot and Rauen, 2007). The preservation solution should also be formulated to the same specifications as the perfusate as inadequate oncotic pressure may lead to oedema formation even before ECP LA has been initiated (Drapanas et al., 1966).

2.3 Initiation ofPerfusion Following Transport and Data Collection

Upon arrival in the laboratory the organ should be connected to the perfusion system as soon as possible to limit the period of ischemia (Fig. 1). An equilibration period with a flow rate that is initially kept low and gradually increased allows the specimen to warm up slowly; this has been shown to be advantageous as it reduces edema formation (Wüstenberg, 2006; Patan et al., 2009).

Organ viability should either be continuously or at the very least measured in short intervals, to permit early intervention and the correction of parameter deviations (e.g. potassium concentration, blood pressure, and pH). Naturally the same applies for measurements in which a development over time is of interest. Due to the nature of the experiments, tissue samples are usually collected following conclusion of the perfusion period, but careful sampling during the perfusion period may also possible.

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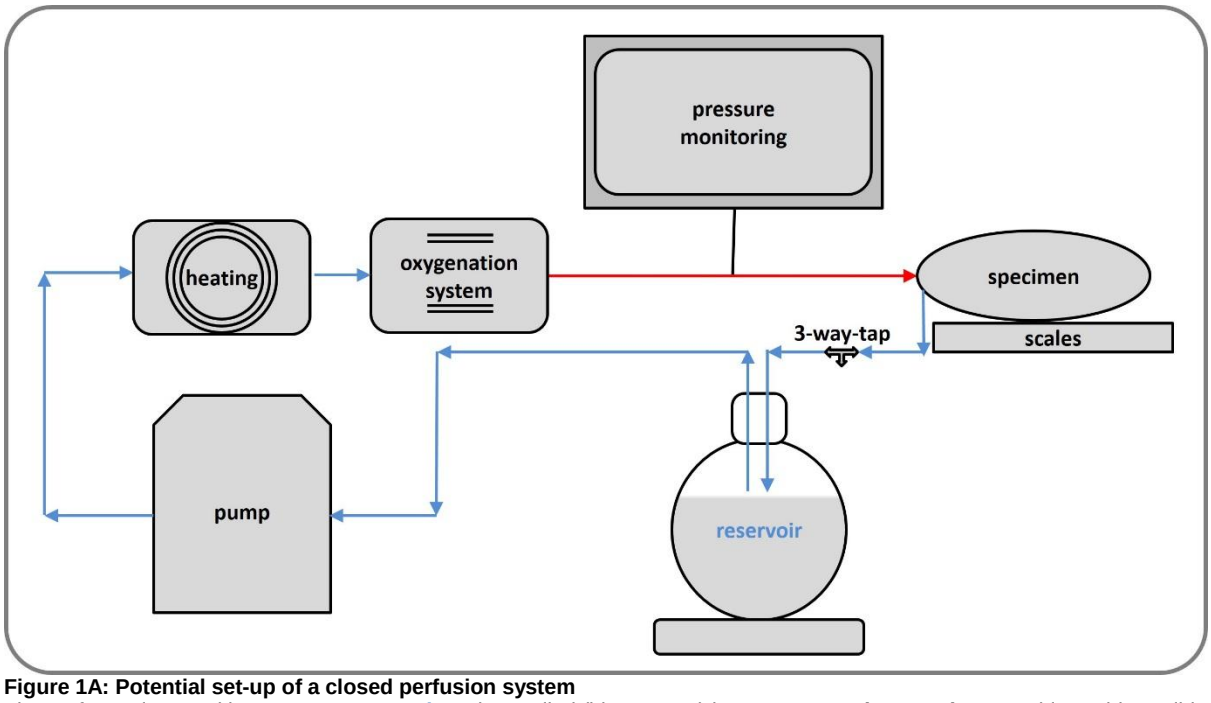

Figure 1A: Potential set-up of a closed perfusion system
The perfusate is stored in a **venous reservoir** and propelled (blue arrows) by a **pump** to a **heat exchanger**, this could possibly be a water bath, which allows the perfusate to be warmed to body temperature. The perfusate flows through an **oxygenation system** e.g. a hollow fibre membrane oxygenator. The now oxygenated perfusate (red arrow) is eventually pumped through the **specimen's** main arteries, capillary network and venous system. A device for measuring the **perfusion pressure** is connected to the circuit. The venous return is fed back into the system via the cannulated main veins of the specimen. To assess oedema formation during perfusion the specimen rests on **scales**. Perfusate samples are taken via **3-way taps** which are incorporated into the tubing.

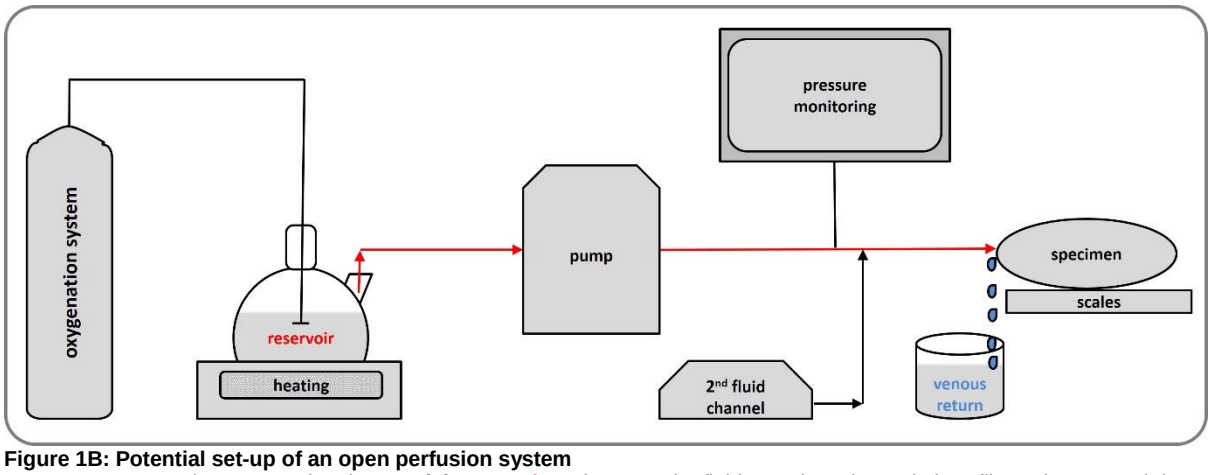

Figure 1B: Potential set-up of an open perfusion system
An **oxygen source** is connected to the **arterial reservoir** and gasses the fluid e.g. via a sintered glass filter. The reservoir is warmed to body temperature by a **heating device**, this might be a water bath or hot plate. A **pump** propels (red arrows) the solution through the main arteries, capillary network and venous system of the **specimen**. The **venous return** (blue drops) drips freely into a beaker and could be collected at that point. Throughout the experiment the leg rests on **scales** to assess weight gain. A **pressure monitoring system** is connected to the system to inform on continuous perfusate pressures. A **second controlled fluid channel** is formed by another pump system and could introduce select additives into the system.

2.4 Perfusate

The choice of the most suitable perfusate is of particular importance and is dependent on the specific research interest, existing resources and availability. While autologous blood is obviously the most appropriate choice, availability, coagulation and the cellular damage created by the mechanical perfusion system limit its use, and other isotonic, isooncotic solutions that adequately sustain physiological functions may be preferred.

Oxygenation

In order to meet the tissues' oxygen demand most perfusion set-ups use gas enriched perfusates. A sufficient oxygen supply guarantees availability of ATP as an aerobic energy source, thereby protecting and sustaining mitochondrial function. As these cell organelles are known to be involved in signaling pathways responsible for apoptosis and necrosis their protection

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leads to a reduction in cellular damage and therefore better preservation and (re)transplantation outcomes(Fuller and Lee, 2007).

Early experiments in isolated perfused caprine, canine and feline udders incorporated isolated perfused lungs in the circulatory system. In this system with the trachea connected to a gas source the perfusate flow is directed from the lung parenchyma to the isolated organ thereby mirroring normal physiology. The maximum perfusate flow rate achieved by this technique compared favourably with in vivo rates of blood flow and was superior to other perfusion experiments at the time (Hebb and Linzell, 1951). However this extensive set up has not been used since and lungs have subsequently been replaced by other means of oxygenation (Hardwick and Linzell, 1960).The simplest and most widely adopted gas enrichment method is to channel gas directly into theperfusate reservoir prior to its circulation through the system. This approach was most likely used in various experiments in which the publications solely mention the use of "gassed" (Kietzmann et al., 1993) or "oxygenated"(Bäumer and Kietzmann, 1999)perfusates without further specification of the oxygenation system. A suitable way to gas perfusate reservoirs is the use of glassware with a sintered glass filter (Tindal, 1957) but a bubble oxygenator, which bubbles (pure) oxygen through the blood reservoir represents an equally effective way (Smith et al., 1985). An example of a slightly more sophisticated method is the use of a rotating disc oxygenator (Drapanas et al., 1966; Cameron et al., 1972) whereby several parallel discs rotate within the reservoir containing unoxygenated blood and the gas exchange occurs at the disc-blood interface. More recently the use of membrane or hollow fibre oxygenators (Butler et al., 2002; Mueller et al., 2013)typically deployed during cardiopulmonary bypass (CPB) surgery or life support (extracorporeal membrane oxygenation) have been reported in perfusion experiments. These systems generally follow the same concept as rotating disc oxygenators as oxygen exchange occurs at a porous membrane. A simple but effective adaptation of this principle is the use of gas permeable tubing exposed to room air in a custom made oxygenation chamber which has been shown to be effective for oxygenation of media containing erythrocytes (Hamilton et al., 1974; Patan et al., 2009).

The by far most frequently used gas is carbogen with a composition of 95% oxygen (O_2) and 5% carbon dioxide (CO_2) as described inKietzmann's model (Kietzmann et al., 1993)). This might be due to the fact that the buffer capacity of commonly used cell free perfusion solutions is dependent on the presence of CO_2(Mancina et al., 2015). In addition to that a higher oxygen content than the one in room air might be necessary in solutions without oxygen carriers in order to achieve the tissues'required O_2 supply. Pure oxygen is rarely used in perfusion experiments (Drapanas et al., 1966) and use of carbogen of varying relative CO_2-concentrations is common place (2.5% (Grosse-Siestrup et al., 2002b) to 7% (Hardwick and Linzell, 1960)). A reason for this might be that unphysiological high oxygen tension may lead to production of free radicals (Fuller and Lee, 2007) and the lack of buffer capacity of CO_2 may result in an excessive pH drop. Occasionally authors describe air as sufficient for gassing the perfusion fluid. These set-ups are usually based on an erythrocyte containing perfusate, such as blood/ plasma (Patan et al., 2009) or blood/ dialysate mixtures (Wagner et al., 2003)and apply a below physiological flow rate (Wagner et al., 2003). For erythrocytes,the body's optimal oxygen carrier room air is adequate to achievesatisfactory saturation of the perfusatewhile a low flow rate may support sufficient time for gas exchange in the oxygenation system. Recently specific perfusion solutions have been tested in the absence of a dedicated oxygenation system. Polyak et al. (2008) perfused equine large colon with either an adapted cell free preservation solution or oxygenated whole blood. Organs perfused with the novel solution relied on ambient air as their sole source of oxygenation.In this report the authors go on to conclude that the novel composition of specific (and partly well established) compounds of the perfusateisnot only capable to prevent oedema (e.g. via modified hydroxyethyl starch, and mannitol) and provide cells with adequate energy (e.g. via ATP intermediates and dextrose) but also leadsto a reduction in the inflammatory response via the addition of prostaglandin E_1 and of L-glutamine positively affecting pathways involved in intestinal stress. L-glutamine may also be responsible for upregulation of heat shock proteins during ischemia, which in turnare associated with cell survival. Vascular and biochemical variables have also been shown to be superior when organs were perfused with the modified solution than with blood (Polyak et al., 2008). This suggests that a well-balanced artificial solution, designed for a specific specimen is able to maintain viability of a perfused organ, potentially even without a dedicated oxygen source. Lacking an oxygenation system may lead to an easier perfusion set-up, but developing and testing a highly complex perfusate is labour and cost intensive and might not be practical in every instance.

Pressure and flow rate

Haemodynamic parameters, namely blood pressure (P, $\Delta P=P_{arterial}-P_{venous}$), blood flow (F), and vascular resistance (R) are interdependent as described by Ohm's Law:

$$F = \frac{\Delta P}{R}$$

While this equation applies to laminar flow conditions and thereby represents a simplified view of normal blood flow it forms the basis for the artificial circuit set-up.

A perfusion can either be controlled by flow rate or by pressure, as the flow rate is adjusted to reach a certain pressure, or vice versa. Most frequently flow controlled set-ups have been used (Chapman et al., 1961; Villeneuve et al., 1996; Grosse-Siestrup et al., 2002b; Zeitlin and Eshraghi, 2002; Dragu et al., 2011), however the latest evidencesuggests that pressure controlled perfusionsare preferred for organ preservation (Mancina et al., 2015).

In flow controlled experiments two strategies may be applied: a low flow or a high flow approach. In low flow circuits the average flow rate is below physiological, therefore aiming for a lower than in vivo perfusion pressure. Despite this reduced flow a functional perfusate flow, which fully reaches and supplies the capillary bed, could be demonstrated by the use of dye solutions (Kietzmann et al., 1993). Specimens seemed well preserved and remainedviable for up to twelve hours (Cypel et al., 2008; Mueller et al., 2013). Low flow rates comparedfavorably to high flow experiments based on histologically evident microvasculature impairment and subsequent hydrostatic oedema formation (Cypel et al., 2008; Constantinescu et al., 2011; Mueller et al., 2013). Systems which employ a high flow approach aim for physiological flow rates and subsequently perfusion pressures, mimicking the in vivo situation more closely.As a higher flow rate leads to lower

organ resistance, this indicates the integrity of blood vessels (Barthel et al., 1989; Bristol et al., 1991; Wagner et al., 2003). Lactate levels in venous perfusate samples obtained during high flow experiments are lower than in low flow experiments and this could also imply a better oxygenation of tissues (Wagner et al., 2003).

Although early experiments on hypothermic perfused kidneys suggest that there is no difference in pulsatile versus constant flow (Pegg and Green, 1976)other groups have shown pulsatile flow to be superior compared to a steady flow of perfusate(Vang and Drapanas, 1966; Finn et al., 1993; Sezai et al., 1999). In cardio-pulmonary bypass experiments pulsatile perfusion,using hydraulically driven dual-chamber physiological pulsatile pumps, enabled better cerebral blood flow with lower resistance than with traditional roller pumps. This might be due to higher haemodynamic energy being generated in the pulsatile perfusion (Ündar et al., 2002). Overall a true pulsatile perfusion reflects the physiological situation better than non-pulsatile set-ups.Nevertheless, the use of roller pumps might still be valuable for research applications as they produce good results in ECP LA and allow more cost effective set-ups.

Temperature

Regarding temperature control perfusatesmay be hypothermic or normothermic.

Hypothermic perfusionsare by enlargeadopted for isolated organ and limb perfusions with the aim to promote organ preservation and facilitate replantation (Smith et al., 1985; Domingo-Pech et al., 1991; Guarrera et al., 2004a, b; Constantinescu et al., 2011; Mueller et al., 2013). Hypothermia slows down cellular metabolism and therefore oxygen consumption, allowing lower flow rates and perfusates without specific oxygen carriers to still meet metabolic requirements (Fuller and Lee, 2007). The possibility to use lower flow rates may result in better preserved microcirculation (Cypel et al., 2008) and might therefore be responsible for less oedema formation reported with hypothermic perfusions(Constantinescu et al., 2011). Other authors however describe weight gain and increased organ resistance due to uncharacteristic sheer forces and viscosity of cold solutions as drawbacks of hypothermic pressure-driven flow rate perfusions(Fuller and Lee, 2007). This weight gain however might be the result of several components working together rather than being solely attributed to the hypothermic perfusion itself. Low temperatures may also lead to reduced antioxidant defenses and subsequently to production of free radicals and toxic concentrations of superoxides within tissues(Fuller and Lee, 2007).In contrast,it is known from extracorporeal perfusion during cardiopulmonary bypass that hypothermia eases inflammation in the brain (Schmitt et al., 2007). During hypothermia neutrophil and monocyte migration is markedly reduced (Biggar et al., 1984), which undoubtedly reduces inflammatory responses but might also lead to a higher infection rate.

Reduced metabolism and altered inflammatory responses may render a hypothermic approach less suitable for most research applications. Indeed normothermia is pursued in almost all scientific implementations of the methodology(Kietzmann et al., 1993; Patan-Zugaj et al., 2012). For studies with a physiological, pharmacokinetic or pharmacologic focus(Roets et al., 1979b; Bäumer et al., 2002; Friebe et al., 2013) the use of hypothermia would seem contra-indicated.

Blood based solutions

In its complex composition blood provides perfectly balanced components as well as the most suitable oncotic pressure; this is reflected in relatively low weight gain from oedema formation in experiments using whole blood as perfusate(Patan et al., 2009; Constantinescu et al., 2011; Mancina et al., 2015). One report shows a higher weight gain in limbs perfused over five hours with diluted autologous blood (blood: Tyrode solution 4:1) compared to ones perfused over eight hours with adapted Tyrode solution containing sodium carboxymethyl cellulose (0.15g/L)(Friebe et al., 2013).Besides its ideal oncotic characteristics whole blood contains erythrocytes which represent an optimal oxygen carrier and therefore enable sufficient oxygen transport to perfused tissues (Mancina et al., 2015).

Instead of autologous blood perfusions several experiments have utilized homologous blood or blood components instead with no apparent negative effects (Hebb and Linzell, 1951; Cameron et al., 1972; Butler et al., 2002).While this may simplify the experimental set-up and potentially reduce inter-animal variability, it is plausible that incompatibility reactionscould occur thereby affecting outcome variables. Until further evidence for this approach can be provided use of autologous blood may therefore be preferable.

Besides obvious advantages with autologous blood perfusions, slightly greater data variability may be encountered compared to perfusions with fully artificial solutions, due to inter-individual differences in the animals' blood composition (Tab. 1).

Unmodified blood is not suitable for perfusion purposes, as at least the addition of anticoagulants is necessary for its use. It may also be recommended to dilute whole blood which decreases its viscosity and in turn leads to an improved blood flow. This applies particularly to the capillary bed, where blood flow is comparatively slow and shear rates are low. As blood is a shear thinning non-Newtonian fluid its viscosity is dependent on the extent of present shear rates. Where shear rates are low (as in the capillary bed) blood viscosity is highest. So despite a reduced concentration of oxygen carriersdue to its dilution, the improved flow rate leads to better tissue supply and consequently to superior organ function (Dittrich et al., 2000). For dilution purposes, plasma (Patan et al., 2009) or artificial solutionssuch as Tyrode solution (Friebe et al., 2013), modified Krebs Henseleit buffer (Mancina et al., 2015) or dialysate solution (acetate hemodialysis concentrate HD 22, Fresenius Medical Care, Bad Homburg, Germany) (Wagner et al., 2003)have been used successfully.

Disadvantages associated with the use of whole bloodmainly pertain to the logistical challenges associated with the harvestand processing to render it a suitable perfusate; this is certainly more demanding as well as more time-sensitive compared to the preparation of cell free solutions. Furthermore, stress that animalsmay experience ante mortemcould change theblood composition; one example of this being the adrenalin related haemoconcentration in horses(Persson, 1967). If autologous blood is to be used it is typically harvested at the time of death, requiring both blood and tissue specimens to be processed at the same time. For homologous perfusions, blood donors may be utilized independent from the euthanasia of organ donors. Alternatively, in the case of autologous perfusions, blood could be collected slightly prior to euthanasia.

Irrespectively, the limited volume of blood availablemight require use of a recirculating system to ensure perfusion at adequate flow rates and over the desired length of time. In this case it should be considered that closed or recirculating systems arealso associated with damage to blood cells (Watanabe et al., 2007; Lee et al., 2007) and metabolic waste products such as lactate will also accumulate over time (Bristol et al., 1991). Furthermore the use of blood collected from animals under anaesthesia or following euthanasia and consequently containing drug residues may affect blood physiology (Barlow and Knott, 1964; Gentry and Black, 1976; Honkanen et al., 1995). Therefore use of blood obtained via exsanguination as part of slaughterwould seem the mostadvantageous option. A drawback however is the potential lack of sterility that occurs during the harvesting process, which might eventually lead to a bacterial contamination, especially in longer experiments.

Table 1: Variousapplicationsforbloodbasedandartificalperfusionfluids
The choiceof a specificperfusion fluid depends on theperfusedorganandtheresearchquestioninvestigated. Care must betakentoselecttheperfusion fluid thatbestservestheintendeduse. This tablegives a shortoverviewofselectapplicationsofeitherbloodbasedorartificialperfusionsolutions. Specificrecipes, compoundsand additives tooptimisethefluid'sperformancearesubjecttorespectivepublications.

Perfusion fluid	Organ	Species	Research Area	Research Group/ Select Publications
Blood based	Heart	Porcine	Pathophysiology	Janse et al., 1980
	Kidney	Porcine	Organ preservation	Grosse-Siestrup et al., 2002a
	Limb	Equine	Pathophysiology	Patan-Zugaj et al., 2012, 2014; Patan et al., 2009; Gauff et al., 2013, 2014
		Porcine	Pharmacology	Wagner et al., 2003
	Liver	Bovine	Organ preservation	Chapman et al., 1961
		Porcine	Perfusion	Adham et al., 1997
				Butler et al., 2002
				Grosse-Siestrup et al., 2002a, b
			Physiology	Cameron et al., 1972
				Drapanas et al., 1966
	Lung	Porcine	Organ preservation	Erasmus et al., 2006
	Udder	Bovine	Physiology	Cowie et al., 1951; Verbeke et al., 1959; Lauryssens et al., 1959; Lauryssens et al., 1960; Lauryssens et al., 1961; Wood et al., 1965
		Caprine	Physiology	Hebb and Linzell, 1951; Hardwick and Linzell, 1960; Hardwick et al., 1961, 1963; Hardwick, 1965, 1966; Linzell et al., 1967; Verbeke et al., 1957, 1968, 1972; Roets et al., 1974, 1979a, b
Artificial	Heart	Porcine	Perfusion	Chinchoy et al., 2000
			Physiology	Araki et al., 2005
				Coronel et al., 1988
	Intestine	Equine	Organ preservation	Polyak et al., 2008
		Porcine	Physiology	Hansen et al., 2000, 2004
				Rehfeld et al., 1982
	Kidney	Porcine	Pathophysiology	Köhrmann et al., 1994
			Organ Preservation	Mancina et al., 2015
	Limb	Equine	Pharmacology	Friebe et al., 2001, 2013
		Porcine	Organ preservation	Constantinescu et al., 2011; Mueller et al., 2013
			Pharmacology	Wagner et al., 2003
	Lung	Porcine	Organ preservation	Cypel et al., 2008
			Physiology	Hellewell and Pearson, 1983
	Udder	Bovine	Pharmacology	Kietzmann et al., 1993, 1995; Forster et al., 1999; Bäumer and Kietzmann, 1999, 2001; Ehinger and Kietzmann, 2000a, b; Ehinger et al., 2006; Kietzmann et al., 2008, 2010; Schumacher et al., 2011

ALTEX Online first
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Cell free solutions

Cell free perfusion solutions are generally based on Ringer's isotonic solution containing sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), and sodium bicarbonate ($NaHCO_3$) as elementary ingredients. The solutions form the basis for the perfusate and are usually modified and enriched with various additives. The most commonly used variants for research purposes are Tyrode solution (Kietzmann et al., 1993), Krebs Henseleit solution (Berhane et al., 2006) and bicarbonated Krebs Ringer solution (Hansen et al., 2004) (Tab. 2). Tyrode solution used for organ perfusion was associated with good tissue viability of isolated perfused digits (Friebe et al., 2001). It was also successfully used in studies focusing on potential mucous membrane irritation of Lugol's iodine solution for the treatment of endometritis (Bäumer et al., 2002) and in studies on pharmacokinetics in the context of joint disease (Friebe et al., 2013). In support of therapeutic organ transplantations several specifically designed perfusion solutions have been reported; most commonly used are Eurocollins solution and the University of Wisconsin solution. The former distinguishes itself by its relatively high glucose content (214mM/L) and is best suited for lung preservation (Hicks et al., 2006), while the latter is characterizedby three impermeants (lactobionate, raffinose, and hydroxyethyl starch) and is well-matched for kidney, liver and pancreas preservation (Hicks et al., 2006). Both of these solutions have been shown to be unsuitable for the preservation of muscle and extremities(Tsuchida et al., 2001, 2003). As mentioned earlier Polyak et al. (2008) demonstrated that the use of a novel unoxygenated artificial solution they had developed could maintain viability of equine intestine in perfusion experiments for twelve hours and was superior to the use of whole blood (Polyak et al., 2008).

One of the advantages associated with the use of cell free solutions is their relatively easy handling in a perfusion set-up when compared with blood. The solution is readily available, even in large amounts that might be needed if an open system is the set-up of choice and variability in perfusate composition is not a concern. The use of cell free solutionsfacilitates standardization of the experimental environment, minimizes unwanted interactions and allows a refined focus on thebehaviour of specific cell types. A distinct disadvantageis represented by increased oedema formation due to inadequate oncotic pressure of artificial perfusion solutions (Domingo-Pech et al., 1991; Zeitlin and Eshraghi, 2002). In unpublished data by the group weight gain was indeed a concern but doesn't exceed values reported by other groups. Nonetheless, weight gain of organs has also been reported in experiments using whole blood as perfusate (dilution blood: Tyrode solution, 4:1)(Friebe et al., 2001).

Table 2: Basic compositions of commonly used solutions

Recipes vary between different research groups and suppliers. For perfusion experiments mixtures are often adapted to closely represent the plasma concentration of respective components of the species investigated.

Component in mM/L	Tyrode	Krebs Henseleit	Krebs Ringer (bicarbonated)
NaCl	137	118	115
KCl	2.7	4.7	5.9
$CaCl_2$	1.8	1.3	-
$MgCl_2$	1.05	-	1.2
$NaHCO_3$	12	25	25
$MgCl_2$	1.8	-	-
NaH_2PO_4	0.42	-	1.2
KH_2PO_4	-	1.2	-
$MgSO_4$	-	1.2	-
Na2SO4	-	-	1.2
Glucose	5.5	11	10

Additives

In order to optimize the perfusion's performance a broad variety of additives has been used, and the most commonly used additives in the context of experiment types, specimens and species are presented here (Tab. 3). Additives that are infrequently used and that are highly specific to organs or research questions are not covered.

The use of whole blood perfusates requires the addition of an appropriate anticoagulant for the prevention of thrombus formation and consequent disturbances in blood flow; furthermore, anticoagulants might ease the inflammatory response associated with ischemia/ reperfusion injury, as clotting plays an important role in this process (Blaisdell, 2002). For this purpose heparin, an inhibitor of antithrombin III (Hirsh et al., 1995), is the most widely used agent (Hardwick et al., 1961; Patan et al., 2009; Constantinescu et al., 2011). In addition to volume expansion, dextran solutions also contribute thrombolytic and antithrombotic properties (Fischer et al., 1985; De Raucourt et al., 1998) rendering them popular additives toperfusates(Tindal, 1957; Drapanas et al., 1966; Cameron et al., 1972; Rehfeld et al., 1982; Domingo-Pech et al., 1991; Kietzmann et al., 1993; Hansen et al., 2000).

ALTEX Online first
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Table 3: Most commonly used additives in perfusion experiments

Additive	Class	Function
Heparin	Anticoagulant	Prevention of thrombi
Dextran	Anticoagulant Impermeant	Prevention and lysis of thrombi Prevention of oedema
Mainly: **Penicillin** *and* **Streptomycin**	Antimicrobial agents	Prevention of bacterial overgrowth
Sodium bicarbonate	Buffer	Maintenance of pH levels
Methylprednisolone	Glucocorticoid	Prevention of general inflammation (reduction of vascular leakage)
Plasma proteins/ Serum albumin	Impermeant	Prevention of oedema
Glucose	Impermeant Metabolic substrate	Prevention of oedema Maintenance of metabolism
Aminoacids	Metabolic substrate	Maintenance of metabolism
Erythrocytes	Oxygen carrier	Enhancement of oxygen delivery and maintenance of aerobic metabolism
Cell free solutions	Rheological active agent	Haemodilution
Mainly: **Nitroglycerin** *or* **Prostacyclin**	Vasodilator	Improvement of vascular flow

Vasoconstriction, a core response in reperfusion injury,may be counteracted by the addition of vasodilators such as nitroglycerin (Domingo-Pech et al., 1991) or prostacyclin (Erasmus et al., 2006)which consequently improve flow rates. Especially in udder perfusion studies, when aspects of milk production are in the center of interest and relatively high flow ratesare required, blockage of 5-hydroxytryptamine induced vasoconstriction has been shown to be advantageous (Roets et al., 1974, 1979a, b). An option to improve flow rates, especially when using whole blood perfusates, is altering the perfusate's rheological properties. This prompted research groups to haemodiluteperfusates to modify blood viscosity and reduce vascular resistance, a technique widely applied in cardiopulmonary bypass procedures(Dittrich et al., 2000). As eluded to above this approach improves tissue oxygenation despite a reduced concentration of erythrocytes, due to advanced circulatory function(Dittrich et al., 2000). Reported diluents have been cell free solutions (Domingo-Pech et al., 1991; Wagner et al., 2003; Erasmus et al., 2006; Friebe et al., 2013)or autologous plasma (Patan et al., 2009; Patan-Zugaj et al., 2012, 2014) which comes with the added benefit of increasing oncotic pressures. Ascell free solutions are associated with low oncotic pressuresresulting in oedema formation and weight gainadding plasma expanders such as plasma proteins (Verbeke et al., 1968; Roets et al., 1974; Patan et al., 2009) and purified albumin (Rehfeld et al., 1982; Barthel et al., 1989; Riviere et al., 1989; Brunicardi et al., 2001)may counteract oedema formation. Plasma expanders/replacers, used for clinical and research purposesand showing a similar effectare dextran (Cameron et al., 1972; Kietzmann et al., 1993), cellulose (Friebe et al., 2001), hydroxyethyl starch(Mueller et al., 2013)andmannitol(Domingo-Pech et al., 1991; Labens et al., 2013). While mannitol isconsidered an inert substance, recent research has suggested possible interdependencies with monocyte and neutrophil function (upregulation HLA-DR in monocytes, upregulation of CD11b in neutrophils and monocytes, and inhibition of neutrophil apoptosis (Turina et al., 2008)). Hydroxyethyl starch (Handrigan et al., 2005; Matharu et al., 2008) and cellulose (Hänsch et al., 1996; Moore et al., 2001; Bae et al., 2004; Ewoldt et al., 2004; Hernández et al., 2004, 2009) have also both been shown to alter inflammatory responses due to their different effects on neutrophils. While glucose is also able to support the maintenance of oncotic pressures(Hicks et al., 2006)it is responsible for the specimen's metabolism and survival.

Glycolysis represents the main energy source for living tissue and ensures ATP production under anaerobe and aerobe circumstances (Silbernagl, 2012) and is indeed consumed by specimens in perfusion experiments. Glucose is therefore an often used additive in blood based solutions (Butler et al., 2002; Patan et al., 2009) and part of the basic recipe in artificial fluids. Closed systems require a regular compensation for glucose loss.

To ensure aerobe metabolism, sufficient oxygen supply is essential, and oxygen carriers are often added to artificial solutions. Isolated erythrocytes as the body's natural oxygen carrier are very well suited for this purpose although their use in closed systems entails the risk of mechanical cell damage over time (Watanabe et al., 2007; Lee et al., 2007). In early experiments fluorocarbons have been tested withequivocal results (Usui et al., 1985; Smith et al., 1985).

Metabolic activity is also influenced by the perfusate's pH and vice versa. Acidosis which might occur due to hypoxemia or hypoperfusion leads to an increased level of lactic acid (Silbernagl, 2012).This is initially buffered by alkalic substances such as $NaHCO_3$, and because of this such substances are also ingredients of perfusates(Cameron et al., 1972; Riviere et al., 1989; Domingo-Pech et al., 1991; Mancina et al., 2015). Some authors report on gas flow rate to adjust partial pressure of carbon dioxide (pCO_2) levels and pH(Cypel et al., 2008). In most previous perfusion set-ups however precise monitoring and adaptation of gas flow rates and CO_2 content are technically challenging.

To fight bacterial overgrowth in perfusates and specimens, antibiotics have been used in select applications. Most commonly a standard combination of β-lactam antibiotics (usually penicillin (Hardwick et al., 1961; Riviere et al., 1989; Bristol et al., 1991)) and aminoglycosides (usually streptomycin (Hardwick et al., 1961) or gentamicin (Riviere et al., 1989; Bristol et al., 1991)) were chosen. Drugs were applied via the perfusate either constantly (Riviere et al., 1989; Domingo-Pech et al., 1991) or intermittentlyat predetermined time intervals(Butler et al., 2002). Many studies however have been successful without the use of antibiotics, and this was documented in one study, where no bacterial contamination was found for up to 24 hours; neither in the specimen nor in the whole blood perfusate(Chapman et al., 1961). But as the harvesting process and the system used were sterile, bacterial contamination is still considered a concern because most perfusion experiments are conducted in an unsterile environment.

Almost all perfused specimens suffer some time of warm and/ or cold ischemia. It is known that reperfusion of ischemic tissue leads to endothelial damage which is involved in vascular ischemia/ reperfusion injury (Blaisdell, 2002). It is either mediated by IgM natural antibodies (Chan et al., 2004) or by activation of the classical complement pathway by apoptotic endothelial cells (Mold and Morris, 2001). This eventually leads to vascular leakage and hence to oedema formation in perfusion experiments. CPB surgery is also known to trigger complement system and cytokine production (Butler et al., 1993). To suppress this cascade the use of glucocorticoids is widely accepted during CPB (Varan et al., 2002) and during perfusions experiments with an interest in organ preservation methylprednisolone is used(Domingo-Pech et al., 1991; Constantinescu et al., 2011)with the common aim to reduce the synthesis of inflammatory cytokines and the associated development of hydrostatic oedema.

2.5 Viability measurements

To monitor the specimens' viability during perfusion several parameters can be measured. The following gives an overview over available viability data, which can be applied to all specimens. In addition, organ specific measurements may be carried out to obtain further detailed information about viability and tissue function, e.g. milk synthesis (Hardwick and Linzell, 1960), bile production (Drapanas et al., 1966), responsiveness to electro-stimulation (Constantinescu et al., 2011), or endocrine response (Jensen et al., 1978). In general, metabolic values are tissue specific and dependent on the mass of the perfused organ. A simple definition of the overall cut off value for viability can therefore not be given and comparisons between studies are to be made cautiously.

Oedema

Prolonged ischemia causes alterations in the capillary bed which ultimately leads to an increased vascular permeability and therefore oedemaformation (Blaisdell, 2002). Weight gain of perfused specimens thus reflects the degree of ischemia/ reperfusion injury (Petrasek et al., 1994; Adham et al., 1997) and the integrity of the microvasculature (Mueller et al., 2013). Excessive oedemaformation results in rapidly progressive deterioration of organ function, which renders it unsuitable for transplantation and research alike(Verbeke et al., 1972). Next to reperfusion injuryoedema formation is also promoted byinadequateoncotic pressures,perfusion pressures, and flow rates.

The easiest way to monitor oedema formation is by weighing specimens before and after perfusion. While histologic assessments will also inform on oedema formation other parameters such as the measurement of skin fold thickness or the wet to dry weight ratio of harvested tissue samples have all been used.

Hardwick and Linzell(1960) defined oedema as a greater than 20% weight gain which occurred in approximately 50% of caprine mammary gland perfusions. The same authors pointed out that the weight gain often occurred rapidly as a terminal event at the end of five to 27 hour longexperiments(Hardwick and Linzell, 1960). Kietzmann et al. (1993) however expressed concerns that due to milk production in the isolated perfused udder, increased weight might not be a reliable parameter for oedema formation(but might just as well reflect milk production) requiring earlier mentioned parameters (skin fold thickness , histology) to be added. However it was subsequently found that with an average weight gain of 14% over a six hour perfusion period, a concurrent increase in skin fold thickness or change in histologic appearancewas not observed (Kietzmann et al., 1993). Other studies have applied more stringent limits for inacceptable weight gainleading to the exclusion of specimens with a greater than ten percent increase during perfusion (Zeitlin and Eshraghi, 2002).

Perfusion of calf livers with a human blood based perfusate for up to nine hours resulted in mild to moderate interstitial oedemaformation (histologic assessment) which was associated with a total weight gain of approximately 19 to 21%. Interestingly ten percent of the observedweight gain already occurred during the initial wash out period of autologous blood with Ringer's lactated solution (Drapanas et al., 1966). This may indicate that vascular damage or inadequate oncotic pressure was already present at the start of the perfusion experiment.

Different approaches to counteract oedema formation have been discussed earlier; as mentioned above these include hypothermic perfusates, addition of glucocorticoids and the optimization of perfusion conditions (perfusate, flow rate, pressure).

Oxygen

The rate of oxygen consumption by tissue can be determined applying the Fick equation:

$$VO_2 = Q\ x\ (C_aO_2 - C_vO_2)$$

The cardiac output in mL/min is represented by Q and the difference between the arterial (C_aO_2) and the venous (C_aO_2) oxygen content describes the arteriovenous oxygen difference. The arterial oxygen content represents the volume of oxygen carried per 100mL blood and is calculated by:

$$O_2\ carried\ by\ Hb + \ O_2\ in\ solution$$

$$\left(1.34\ x\ Hb\ x\ \frac{S_aO_2}{100}\right) + (0.003\ x\ P_aO_2)$$

The Huefner's constant (1,34) represents the experimentally measured maximum oxygen carrying capacity of haemoglobin. It is multiplied by the haemoglobin concentration (Hb) per 100mL blood and the percentage of oxygen saturated haemoglobin (S_aO_2). The amount of dissolved oxygen is calculated by the product of the partial pressure of oxygen (P_aO_2 in mmHg) and the constant 0.003, representing the amount of oxygen dissolved in plasma. The venous oxygen content can be calculated likewise. In perfusion experiments the organ's weight is often taken into consideration (Chapman et al., 1961; Jensen et al., 1978), e.g. Patan et al. (2009) report on an oxygen uptake of $6.4x10^{-6}$±$8.9x10^{-5}$ L/g/min. Measuring oxygen saturation (SO_2), partial pressure of oxygen (pO_2) and pCO_2 in arterious and venous perfusate samples also sheds light on the oxygen use of the perfused organ (Zeitlin and Eshraghi, 2002; Wagner et al., 2003; Polyak et al., 2008; Constantinescu et al., 2011).

Glucose

In living cells the metabolic pathway of glycolysis converts glucose to pyruvate. The free energy released in that process is used to produce the energy sources ATP and nicotinamide adenine dinucleotide (NADH). In ECPLA consumption of glucose contained in theperfusatewould imply a functional glycolytic metabolism, producing energy to keep the specimen alive, but could however also be contributed to bacterial glucose consumption in a contaminated set-up. It has been suggested that a glucose utilization of ≥200mg/h is indicative of a viable distal equine digit(Friebe et al., 2001). It is however difficult to extrapolate this to other experiments as glucose metabolism is tissue specific and also dependent on the mass of the perfused organ. In closed systems the consumption of glucose over time can be assessed by comparison of glucose levels in the arterial reservoir at different time points. To replenish glucose it is either added directly to the perfusate(Riviere et al., 1989; Wagner et al., 2003)or the arterial reservoir of the perfusateis replacedat regular intervals thereby re-establishingprior glucose levels (Patan et al., 2009). In open systems glucose consumption may be assessed comparing arterial reservoir contents with glucose content of the venous return from the specimen.

Lactate

Lactate production is increased during anaerobic glycolysis and therefore a suitable parameter to inform on the adequacy of the oxygen delivery to tissues.In perfusion experiments an elevated lactate concentration in the outflowing perfusate or venous return might be observed immediately after the limb is connected to the circuit.This is likely due to accumulation of lactate during anaerobic transport conditions which at commencement of perfusion is cleared and washed out(Kietzmann et al., 1993). Increases in lactate concentrations have also been observed after arterial reservoir exchanges resulted in a period of relatively low O_2 saturation of the perfusatedue to thenew reservoir solutionnot yet being fully oxygenated (Patan et al., 2009). Friebe et al. (1993) postulate in their minimum requirements for viability of the equine distal limb a lactate production of ≤400mg/h, a criterion met by their and other groups'experiments(Patan et al. 2009). Again these values are tissue and mass dependent and can't be seen as absolute references. Both groups report on physiological lactate levels, even if an increase of lactate levels within the physiological range was noted over the course of the experiments (Friebe et al., 2001, 2013; Patan et al., 2009; Patan-Zugaj et al., 2012, 2014). In contrast lactate levels were (constantly) elevatedin the venous perfusatein porcine isolated limb perfusions (up to 105mg/dL(Wagner et al., 2003), mean maximum value 19.59mmol/L (Constantinescu et al., 2011))likely due to the higher metabolic demands ofa greater amount of included soft tissues.To summarize, due to the lack of hepatic clearancea rise in lactate levels can be observed in the majority of perfusion experiments over time, even if measurements do not exceed the physiological range by the end of the experiment (Smith et al., 1985; Riviere et al., 1989; Adham et al., 1997; Polyak et al., 2008).A small decrease of lactate levelsmay be observedafter several hours of perfusion which may be explained by modest lactate utilization by myocytes (Constantinescu et al., 2011).

Potassium

Potassium levels in the perfusatemay be used as an indirect measurefor cell integrityand cell death (Ward and Buttery, 1979; Bortner et al., 1997). Potassium concentrations are higher in the intracellular space due to energy dependent active transport systems (Na^+/K^+ATPase) maintainingan osmotic balance. In the dying cell potassium follows its electrochemical gradient to the extracellular space (Bortner et al., 1997; Trimarchi et al., 2000), thereby leading to a measurable increase in potassium in the perfusate(Polyak et al., 2008; Patan et al., 2009; Constantinescu et al., 2011). On the other hand intracellular potassium transport indicates availability ofadequate energy suppliessustained by the perfusion circuit (Patan et al., 2009). A potassium efflux of ≤ 7% has been proposed to indicate adequate cell viability during ECP LA (Ward and Buttery, 1979). In order to control potassium levels in perfusion experiments an insulin/ glucose solution may be added to the perfusate(Constantinescu et al., 2011). Insulin increases the activity of the Na^+/K^+ ATPase pump and excess potassium is removed from the extracellular space. However other effects of insulin should be noted, e.g. change in endothelin-1 expression and associated laminitic changes in the perfused equine digit (Gauff et al., 2013, 2014). In the event of acidosis during perfusion experiments potassium may also move from the intracellular space to the extracellular fluid in an attempt to buffer dropping pH levels(Oster et al., 1978). In this case an increase of potassium levels in the perfusate is initially not elicited by cell death and has to be assed in the context of the metabolic imbalances.

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme responsible for the interconversion of lactate and pyruvate and thus involved in anaerobe glucose metabolism and glucose biosynthesis (Berg et al., 2013). It can be found in almost every tissue with a tissue specific distribution pattern of its isoenzymes (Markert and Ursprung, 1962) with the isoforms LD1 and LD2 being dominant in erythrocytes (Kato et al., 2006). Therefore elevated LDH activity may be associated with haemolysis and cell injury/death in general (Legrand et al., 1992). In perfusion experiments it has consequently been used as an indicator of cell death following hypoxemia or haemolysis (Ward and Buttery, 1979; de Lange et al., 1992; Moen et al., 1994). In whole

blood perfusion experiments of the equine digit, utilizing a closed system Patan et al. (2009) reported on LDH levels in the range of 12.0±14.7U/h in the isolated perfused equine digit and the authors observed an albeit statistically insignificant increase in LDH levels over a perfusion period of up to ten hours(Patan et al., 2009) indicating a good viability of the specimen.In open systems using Tyrode solution based perfusates LDH levels tended to be lower (Kietzmann et al., 1993; Friebe et al., 2001, 2013)presumably due to the lack of LDH containing erythrocytes and have been reported to indicate adequate tissue viability of an equine distal limb during perfusion if below 10U/h (Friebe et al. 2001).

3 History and Important Findings

3.1 Transplantation, Replantation and Preservation

Organs

In the context of transplantation surgery prolonged preservation using pulsatile perfusion has been shown to be superior to simple cold storage when assessing graft and patient survival, rate of delayed graft function and the need for post-transplant dialysis (kidney transplant) (Peter et al., 2002; Shah et al., 2008). Standardization of perfusion set-ups was possible by the availability of commercially available perfusion systems (e.g. Waters RM 3, MOX-100, (Guarrera et al., 2004a)). This and the augmentation of perfusion solutions (Belzer MPS/ Vasosol MPS) with vasodilators (nitroglycerin, and prostaglandin E1), antioxidants (polyethylene glycol-superoxide dismutase, and N-acetylcysteine), Krebs cycle intermediates (α-ketoglutarate) and metabolic substrates (L-arginine) (Guarrera et al., 2004a, b) improved results with organ preservations dramatically.To further optimise this methodology recent studies have evaluated the differences between pressure- and flow-controlled perfusions and concluded that pressure controlled perfusions are superior with reference to renal haemodynamics and acid-base homeostasis (Mancina et al., 2015).

Limbs

The preservation of limbs represents a specific challenge due to the broad range of involved tissues and their variable tolerance to ischemia (Blaisdell, 2002; Constantinescu et al., 2011).

In early ILP experiments performing canine hind limb autotransplants the best outcome was observed implementing continuous hypothermic perfusion with solutions containing fluorocarbon as an oxygen carrier(Usui et al., 1985). Despite these initial positive results fluorocarboncontaining solutions revealed only moderate success during clinical trials of human digital replantation following perfusions(Smith et al., 1985). Fluorocarbon is also known to reduce neutrophil infiltration, which might be an interesting characteristic in organ preservation but could affect outcomes in research applications of the perfusion model(Forman et al., 1992).

Another study was able to demonstrate that ILP over a period of 24 hours maintained viability of amputated canine hind limbs so that after six hours post replantation limbs appeared healthy and peripheral vessels were well perfused. However, significant oedema formation (20 to 50% weight gain) was observed in perfused legs requiring subsequent cooling of the perfusate and use of peripheral vasodilators and steroids (Domingo-Pech et al., 1991) to counteract inflammatory processes (Biggar et al., 1984).

In order to optimize the experimental set-up Tsuchida et al. (2001) assessed different perfusion pressures and solutions. Comparing replantation of amputated rat hind limbs after perfusion with the superior set-up (University of Wisconsin solution, high perfusate pressure) with replantation of non-perfused limbs, outcomes with perfusion were only slightly superior. An observation that might be explained bythe potential deterioration of vascular endothelial function and consequently blood flowwith the use of University of Wisconsin solution (Tsuchida et al., 2003).

Recent preservation experiments in porcine limbs using autologous blood in hypothermic set-up with sub-physiological perfusion pressure have proven the technical feasibility and the great potential of this approach (Constantinescu et al., 2011). This is further supported by Mueller at al. (2013) who were able to replant porcine front limbs after an extracorporeal perfusion time of twelve hours with only minimal impact on ischemia/ reperfusion injury as assessed by histopathology, markers for inflammation and endothelial cell activation. In order to reduce capillary leakage methylprednisolone was added to the perfusate as was done in several other studies (Mueller et al., 2013).

3.2 Research Models

Udder

The isolated perfused udder of various species, but predominantly ruminants, has been widely used in the study of milk synthesis and ejection. It contributed to the understanding of lactation and had impact on the field of dairy research. It has also been used as a model for skin absorption in studies with a pharmacology focus (Tab. 4).

Use of the isolated cow's udder gland is largely based on a technique described by Peeters and Massart(Peeters and Massart, 1952). For this, abattoir derived specimens were perfused with normothermic oxygenated heparinized homologous blood for approximately two hours at a constant pressure. Different radioactively labelled substrates were added to the perfusate and their content in milk, venous return and tissue was recorded (James et al., 1956; Verbeke et al., 1959; Lauryssens et al., 1960). The O_2 uptake as an indicator of viability compared well to the in vivo activity during the first two hours. Despite physiological O_2 consumption, milk yield declined rapidly thereafter resulting in less than normal milk production (Lauryssens et al., 1961).

Taking into consideration that the milk production is dependent on a relatively high flow rate which could not be achieved in these early experiments Hebb and Linzell(1951) included the isolated perfused lungs in the circulatory system as it is known that the passage of shed blood through the lungs reduces its vasoconstrictive properties (Eichholtz and Verney, 1924; Newton, 1933). Others have included vasodilators (dibenamine or dibenzyline) to improve flow rates (Tindal, 1957).

Hebb and Linzell'scomplicated techniquewas consequently replaced by the availability of artificial oxygenation devices (Hardwick and Linzell, 1960).

With the intent to enhance the viability of the used specimens the udders were no longer obtained from dead slaughtered animals but carefully dissected from live animals under epidural anaesthesia which resulted in a very short period (two to 27min) between the interruption of blood supply and extracorporeal perfusion. Oedema was defined as 20% or more weight gain. Given this definition oedema occurred in about half of the experiments. In most cases once the weight gain exceeded 20% milk secretion ceased within one hour. Nevertheless most secretory cells appeared normal in histology upon completion of the experiment (Hardwick and Linzell, 1960). Addition of an artificial kidney to the perfusion system allowed maintenance of high flow rates for a longer perfusion time. It consisted of ten metres of dialysis tubing in a reservoir of 20L of adapted Krebs solution. This technique has since become more popular(Hardwick et al., 1961, 1963; Hardwick, 1965, 1966; Linzell et al., 1967; Verbeke et al., 1968, 1972; Roets et al., 1974, 1979a, b).

In the 1990s the isolated perfused bovine udder was used for pharmacokinetic research. Kietzmann et al.'s seminal work on this model has supported its application relevant to the treatment of mastitis (Kietzmann et al., 1993). In these studies warmed and oxygenated Tyrode solution was the perfusate of choice(Ehinger et al., 2006; Kietzmann et al., 2010).

The system's potential for skin inflammation studies became evident with the collection of several different data (glucose consumption, lactate production, lactate dehydrogenase activity, and pH in the perfusate, histological examination) all indicative of good skin viability during a six hour perfusion interval. Additional determination of the udder skin-fold thickness demonstrated that no oedema had developed (Kietzmann et al., 1993). Subsequently different anti-inflammatory drugs were tested for their effect on eicosanoid synthesis. They were either administered systemically (= via the perfusate) or applied topically on the skin (Bäumer and Kietzmann, 2001). The perfusion model also served as the basis for studies on the transdermal absorption potential of vitamin E acetate from cosmetic formulations (Lampen et al., 2003).

More recently the isolated perfused bovine udder, perfused with Tyrode solution for five hours, was shown to be useful for preclinical trials into biodegradable magnesium implants (Schumacher et al., 2011).

Heart

The isolated perfused heart is an extensively used ex vivo whole organ research model. It helped to gain better understanding of cardiac physiology (contractile function, blood flow and, metabolism) and pathophysiology (ischemia/ reperfusion injury). Furthermore the model forms the basis for the collection of viable cardiac myocytes or for the measurement of electrical activity (Bell et al., 2011). The technique dates back to Langendorff who in 1897 introduced a model of retrograde perfusion of the isolated mammalian heart via the aorta, later known as the Langendorff heart. For this the aortic root is slipped over a fixed cannula in the perfusion system which is in turn connected to a reservoir containing a gassed and warmed perfusion solution, most commonly a modified Krebs Henseleit solution. The model can either be used under constant flow or constant pressure conditions. It allows investigation of a broad spectrum of physiological, morphological, biochemical and pharmacological parameters and is generally accepted as a model to study drug-induced cardio toxicity and ECG conductivity (Skrzypiec-Spring et al., 2007; Bell et al., 2011). In contrast to this model the isolated heart can also be perfused in the working heart mode, i.e. the perfusate enters the left atrium via the pulmonary vein, flows into the left ventricle and onwards into the aorta (Chinchoy et al., 2000; Araki et al., 2005). The right side of the heart is not included into the latter system as for this a biventricular working heart set-up is necessary, in which the right heart performs a physiological low pressure ejection (Demmy et al., 1992; Chinchoy et al., 2000)

Uterus

Another application of isolated organ perfusion is represented by the use of the bovine uterus for the study of mucosal inflammation (Tab. 4). For inflammation studies a mixed Tyrode/ homologous blood perfusate was used (ratio 1:4), whereas irritancy studies were carried out on only Tyrode perfused uteri. The average weight gain throughout the perfusion period of five hours was reported at 20% for Tyrode-perfused organs with no diminished oedema formation in haemoperfused uteri (Bäumer et al., 2002; Braun and Kietzmann, 2004).

Liver

In 1961 Chapman et al described for the first time a successful isolated liver perfusion in calves of up to 24 hours by cannulating the hepatic artery and portal vein simultaneously. This report demonstrated tissue viability based, amongst other things, on measurements of oxygen consumption, presence of constant blood flows and pressures and only very slight changes in histologic parameters. The authors also postulated that the perfusate (autologous blood diluted with Krebs-Henseleit solution to a final haematocrit value of 25 to 35%) could be maintained free of bacteria for at least twelve hours of perfusion without the addition of antimicrobials (Chapman et al., 1961). A similar approach but using homologous porcine blood with several additives (Tisusol, Rheomacrodex© 5% in dextrose, calcium glucoheptonate, heparin, xanthocholate) was successfully used to model hepatic metabolic clearance (Cameron et al., 1972). Following further improvements to the set-up and the addition of a dialysate circuit, autologous haemoperfusion of the isolated pig liver also revealed good results and was shown to be a promising tool to study whole organ liver function and hepatotoxicity (Grosse-Siestrup et al., 2002b). In contrast to these results Drapanas et al (1966) reported on haemoperfusion of the isolated pig liver, using heterologous blood (human) modified with low molecular weight dextran and heparin. After four hours of perfusion this resulted in mild to moderate interstitial oedema, reduced oxygen consumption and a sample weight gain of about 20%. As discussed above, half of this reported total weight gain occurred during the initial wash out period (20 to 30 minutes) with cold Ringer's solution illustrating the inadequacy of this perfusate solution for maintenance of oncotic pressures (Drapanas et al., 1966). Human liver perfusion with Krebs bicarbonate buffer containing 20% prewashed human or bovine red blood cells, bovine serum albumin, α1-acid-glycoprotein, calcium and glucose, however resulted in normal histological liver architecture after a

perfusion period of approximately five hours. Nevertheless the reservoir contained increased levels of alanine aminotransferase likely indicating endothelial cell damage as a result of ischemic injury (Villeneuve et al., 1996).

Intestine

Few attempts to perfuse isolated large animal intestine have been made. For absorption studies isolated porcine duodenum or ileum was perfused under normothermic conditions with Krebs Ringer bicarbonate solution containing various additives such as washed bovine or human erythrocytes, dextran T70, glucose, amino acids or cyclooxygenase inhibitors. (Rehfeld et al., 1982; Messell et al., 1992; Hansen et al., 2000, 2004). The perfusion set-up byMessell et al. (1992) is based on an open system first developed for pancreas perfusion (Jensen et al., 1975). In this study only perfusion pressure was continuously recorded which was in turn associated with nerve function. No viability related data was offered, but stimulation by various means seemed to have affected intrinsic nerves during the perfusion period of six hours(Messell et al., 1992). Hansen et al. (2000) applied the same model, no specific viability measurements have been documented, but motor activity of the gut appeared normalwith contractions leading to a short increase in perfusion pressure. To inform on intestinal function and pathophysiology in the context of ischemia/reperfusion injury, Polyak et al. (2008) developed a model of isolated equine large colon perfusion. A twelve hours perfusion periodwith a novel organ preservation solution and lacking any method of oxygenation proved superior to the use of oxygenated whole blood when arterial blood pressure, flow and intra-vascular resistance, electrolyte concentrations and mucosal integrity were assessed(Polyak et al., 2008).

Kidney

Efforts to establish and improve techniques for isolated kidney perfusion have been ongoing since 1903 (Pavy et al., 1903) with the main focus on the organ's functional preservation for subsequent transplantation surgery. First successful attempts were made using dog kidneys and normothermic perfusion conditions under physiological perfusion pressure in a closed system with a customised oxygenator supplying an O_2-CO_2-mixture. Two different blood preparations were used and weight gain was reported to be 15±2% (Waugh and Kubo, 1969). Use of Tyrode or barium sulphate ($BaSO_4$) solutions for perfusion were associated with well-preserved histological morphology in experiments in which shockwave induced lesions were investigated (Köhrmann et al., 1994). Perfusion with cell free Tyrode solution was on par with autologous blood preparations in terms of reperfusion injury but it was inferior in preserving renal function (Hoechel et al., 2003).

Lung

The isolated perfused large animal and canine lung has been described for physiological modelling. For this purpose either autologous whole blood (Bhattacharya and Staub, 1980) or Krebs' solution augmented with 4,5% ficoll 70 as a colloid oncotic agent (Hellewell and Pearson, 1983)was used as the perfusate. Attempts to establish a set-up for prolonged lung preservation have shown promising results after six hours of perfusion, although impairment of lung function occurred towards the end of the experiment. The perfusate of choice was a blood diluted with Steen® solution (final haematocrit 15%), from which leucocytes and platelets have been eliminated using a Cell Saver (Cat II®; Fresenius Hemocare, Emmercompascum, the Netherlands) (Erasmus et al., 2006). A stable lung function for up to twelve hours of perfusion with cell free Steen® solution has been reported later (Cypel et al., 2008).

Limbs

The technique of isolated limb perfusion (ILP) serves a variety of research interest (Tab. 4).

In the context of equine laminitis an open ILP system using gassed Krebs-Henseleit solution was used to show that endothelium-derived nitric oxide modulates the response to vasoconstrictors and is therefore presumably an important regulator of blood flow in the equine digit (Berhane et al., 2006). In the same research field Patan et al. (2009) demonstrated excellent tissue viability of the laminar tissue for a perfusion period of up to ten hours. Physiological, yet constant and not pulsatile, blood pressures were applied and autologous whole blood was used as perfusate(Patan et al., 2009). Employing the described set-up the authors were also able to show the model's ability to respond to an inflammatory stimulus, created by addition of lipopolysaccharide to the perfusate(Patan-Zugaj et al., 2012, 2014).

Another application of ILP is represented by its use in pharmacokinetic studies. In these an ex vivo perfusion model was used to assess drug distribution in the synovial fluid and synovial clearance after both, intra-articular injection and systemic administration via the perfusate(Friebe et al., 2001, 2013). In this context the authors were able to show that following a perfusion period of eight hours with gassed Tyrode solution and sodium carboxymethyl cellulose the intimal layer of the equine fetlock joint capsule appeared unchanged whereas the subintimal connective tissue presented mild signs of oedema. In this study four minimal criteria for an effective ILP of the equine distal digit were postulated, which are: glucose utilization ≥200mg/h, lactate production ≤400mg/h, LDH activity ≤10U/h, skin surface temperature ≥26°C (Friebe et al., 2001). As described earlier these criteria can only be used as reference for research under similar circumstances, as metabolic parameters are tissue and perfusion dependent (e.g. a closed system may lead to accumulation of lactate or the use of blood based perfusates may increase overall LDH levels due to the mechanical cell damage the pump inflicts on cellular blood components) and will also be influenced by the weight of the specimen and the perfusate volume.

Perfused isolated porcine limbs have furthermore been used for transdermal absorption studies due to similarities with human skin. This work included a comparison between low flow (100ml/min) and high flow (230-250ml/min, physiological) perfusions. The investigation suggested that a higher flow rate improved oxygen supply and reduced the risk of oedema formation due to lower organ resistance (perfusion flow/ perfusion pressure) and improved blood vessel integrity. An additional comparison of two different perfusate solutions in the high flow group revealed improved haemodynamic parameters with use of a dialysate solution augmented with bovine serum and autologous erythrocytes over use of a whole blood dialysate mixture (Wagner et al., 2003).

Table 4: Select perfusion models and their use in research

Organ	ECP LA suitable for	Findings	Model developed by
Limb (equine)	Modelling inflammatory responses in the context of laminitis Investigating the role of hyperinsulinaemia in the context of laminitis	Endotoxin plays a role as causative agent for equine laminitis (Patan-Zugaj et al., 2012, 2014) Hyperinsulinaemia alters endothelin-1 expression in the equine laminar tissue which suggests endothelin receptor antagonists as a potentially new class of agents in the treatment of laminitis (Gauff et al., 2013, 2014)	Patan et al., 2009; Gauff et al., 2013
Limb (equine)	Pharmacokinetic studies in the context of joint disease	Acetylsalicylic acid and salicylic acid accumulate in the synovial fluid after systemic administration, despite subsiding systemic levels (Friebe et al., 2013)	Friebe et al., 2001
Limb (porcine)	Prolonged limb preservation for transplantation	Prolonged limb preservation barely influences ischemia/ reperfusion injury, ECP LA is a promising technique for use in transplantation surgery (Mueller et al., 2013)	Constantinescu et al., 2011
Udder (caprine)	Exploring physiological concepts (milk synthesis)	Metabolism of various substrates and their contribution to milk synthesis, e.g. (Hardwick, 1965; Roets et al., 1979b)	Hebb and Linzell, 1951; Hardwick and Linzell, 1960
Udder (bovine)	Pharmacokinetic skin absorption studies Pharmacokinetic studies in the context of mastitis (intra-mammary and systemic administration)	No cytotoxicity was recorded for various delivery systems, some formulations were superior regarding their maximum vitamin E delivery to deeper skin layers (Lampen et al., 2003) Cefquinome exceeds the MIC_{90} values of common mastitis pathogens after a combined systemic and intra-mammary application (Ehinger et al., 2006)	Kietzmann et al., 1993
Udder (bovine)	Exploring physiological concepts (milk synthesis)	Metabolism of various substrates and their contribution to milk synthesis, e.g. (James et al., 1956; Verbeke et al., 1959)	Peeters and Massart, 1952; Lauryssens et al., 1959
Uterus (bovine)	Modelling inflammatory responses	The mucosal irritation potential of Lugol's iodine solution has been shown on the Tyrode-perfused uterus. In addition a haemoperfused uterus has demonstrated its potential as a model for inflammatory responses (Bäumer et al., 2002)	Kietzmann et al., 1993; Bäumer et al., 2002

4 Potential Future Applications and Conclusion

Perfusion of isolated internal organs to preserve them for later transplantation is well established. Nevertheless optimisation of this process has been subject of new research in the field of extracorporeal perfusion research (Mancina et al., 2015). At the same time successful preservation of severed limbs may increase the chances of reattachment to improve the quality of life for the affected individual. In this respect, ILP is therefore a valuable technique for future research in the field of transplantation, replantation and prolonged preservation of limbs (Constantinescu et al., 2011; Mueller et al., 2013).

The usefulness of ECP LA has been proven in many research areas and future applications may also include use of an isolated perfusion model to study cell specific migration towards different stimuliin the context of the respective donor organ. Controlled administration and tracking of specific labelled cell types, via in situ, ex vivo imaging methods represent only a few of the possible advantages ofthis technique. Focusing on the migration of leukocytes would allow the use of ECP as a model for the early stages of inflammation, investigating e.g. irritants or chemoattractants. Furthermore, if an inflammation-like condition can be produced it would represent an interesting opportunity to investigate anti-inflammatory properties of new drugs applied via the perfusion fluid or topically(Friebe et al., 2001, 2013). The authors are currently expanding this approach into the use of isolated perfused limbs to study short term events and interventions in the context of arthritis. Being a hot topic in various research areas during the last decade, mesenchymal stem cells are representing another interesting target cell. The use of ECP LA would facilitate insights into migration and differentiation behaviour of these cells in various tissues.

In veterinary research recent efforts include, besides ILP, perfusion of intestine. With the future aim to study functional changes associated with colic (Polyak et al., 2008) and grass sickness (authors) in the equine patient.As alternative to mesenchymal stem cells the behaviour of equine chorionic girdle cells may also prove to be a fascinating field of study in this species.

So, to conclude,ECP of large animal specimens represents an excellent opportunity to answer research questions inorgan preservation and transplantation, (patho)-physiology and pharmacology. This method may bridge the gap between basic and applied clinical research. Experiments can be carried out in a relatively physiological, standardized environmentat

greater experimental numbers. By obtainingabattoir samples or surplus biological material from other experiments, this method strongly supports the3R principles by reducing, refining and replacing live animal testing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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